Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*

Takayuki Kuraishi[1], Olivier Binggeli, Onya Opota, Nicolas Buchon, and Bruno Lemaitre[1]

Global Health Institute, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

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The peritrophic matrix (PM) forms a layer composed of chitin and glycoproteins that lines the insect intestinal lumen. This physical barrier plays a role analogous to that of mucous secretions of the vertebrate digestive tract and is thought to protect the midgut epithelium from abrasive food particles and microbes. Almost nothing is known about PM functions in *Drosophila*, and its function as an immune barrier has never been addressed by a genetic approach. Here we show that the Drosocrystallin (Dcy) protein, a putative component of the eye lens of *Drosophila*, contributes to adult PM formation. A loss-of-function mutation in the *dcy* gene results in a reduction of PM width and an increase of its permeability. Upon bacterial ingestion a higher level of expression of antibacterial peptides was observed in *dcy* mutants, pointing to an influence of this matrix on bacteria sensing by the Imd immune pathway. Moreover, *dcy*-deficient flies show an increased susceptibility to oral infections with the entomopathogenic bacteria *Pseudomonas entomophila* and *Serratia marcescens*. *Dcy* mutant flies also succumb faster than wild type upon ingestion of a *P. entomophila* toxic extract. We show that this lethality is due in part to an increased deleterious action of Monalysin, a pore-forming toxin produced by *P. entomophila*. Collectively, our analysis of the *dcy* immune phenotype indicates that the PM plays an important role in *Drosophila* host defense against enteric pathogens, preventing the damaging action of pore-forming toxins on intestinal cells.

gut | innate immunity | insect immunity | entomopathogens

Because the gut epithelium is in contact with microorganisms, it must be armed with efficient systems for microbial recognition and control (1). This is also true for insects such as *Drosophila*, which live on decaying matter such as rotting fruits and ingest large quantities of microbes. Some studies have started to investigate the mechanisms underlying the gut defense to bacterial infection in *Drosophila*. These studies have indicated that (*i*) reactive oxygen species (ROS) production through the enzyme Duox, (*ii*) production of antibacterial peptides through the Imd pathway, and (*iii*) maintenance of gut homeostasis through regulation of stem cell activity are all essential elements of the gut defense to infection (2).

Oral ingestion of bacteria induces the rapid synthesis of ROS in the *Drosophila* gut by an NADPH oxidase called Duox (3). Ingested bacteria were shown to persist throughout the intestinal tract of *Duox* RNAi flies, which indicates a predominant role of ROS in the elimination of ingested microbes. Complementary to this ROS response, several antimicrobial peptides (e.g., *Diptericin*) are produced in the gut under the control of the Imd pathway (4). This local immune response is triggered by the recognition of Gram-negative peptidoglycan by the pattern recognition receptor PGRP-LC (peptidoglycan recognition protein LC) (5) and was shown to contribute to host survival upon intestinal infection with several pathogenic bacteria (6–8). Finally, efficient and rapid recovery from bacterial infection is possible only when clearance of bacteria from the gut is coordinated with epithelium renewal to repair damage caused by infection. Epithelium renewal of the *Drosophila* gut is stimulated by the release of the cytokine Upd3 from damaged enterocytes, which then activates the JAK/STAT pathway in intestinal stem cells to promote both their division and differentiation, establishing a homeostatic regulatory loop (9, 10). Interestingly, both Imd pathway activity and epithelium renewal are also stimulated at a basal level by the indigenous gut microbiota (10).

The peritrophic matrix (PM) forms a layer composed of chitin and glycoproteins that lines the insect midgut lumen (11, 12). Although structurally different, it plays a role analogous to that of mucous secretions of the vertebrate digestive tract and is thought to protect the midgut epithelium from abrasive food particles and microbes. Studies in insects have suggested that the PM plays a role in the defense against ingested pathogens. However, most of these studies are based on indirect evidence, such as insect survival analysis after ingestion of corrosive agents that disrupt the PM (13, 14). Diptera such as *Drosophila* have a type II PM that is continuously produced by specific cells of the cardia, a specialized organ at the anterior of the midgut (15). As the PM grows posteriorly, it encloses the food passing through it all along the digestive tract. To date almost nothing is known about PM functions in *Drosophila*, and specifically its function as an immune barrier has never been addressed by a genetic approach. In this study, we show that a protein with a chitin-binding domain, Drosocrystallin (Dcy) contributes to PM formation in *Drosophila* adults. A loss-of-function mutation in *dcy* results in a reduction of PM width and renders flies more susceptible to infections with the entomopathogenic bacteria *Pseudomonas entomophila*. We show that this lethality is due in part to an increased deleterious action of Monalysin, a pore-forming toxin produced by *P. entomophila*. Collectively, our analysis of the *dcy* mutation indicates that the PM plays an important role in *Drosophila* host defense against intestinal pathogens by preventing the action of toxins on gut cells.

Results

Dcy Is a PM Protein Induced upon Oral Bacterial Infection. A microarray analysis indicated that a gene named *drosocrystallin* (*dcy)* encoding a chitin-binding protein is induced in the *Drosophila* adult gut upon oral infection with the Gram-negative bacterium *Erwinia carotovora 15* (*Ecc15*) (16). Real-time quantitative PCR (qPCR) analysis confirmed that *dcy* is up-regulated soon after infection and reaches its maximum 4 h after infection, at a level approximately sevenfold higher than in unchallenged condition (Fig. 1*A*). A previous microarray study indicated that *dcy* expression is not controlled by the Imd pathway (16). In agreement with

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[1]To whom correspondence may be addressed. E-mail: takayuki.kuraishi@gmail.com or bruno.lemaitre@epfl.ch.

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FlyAtlas (17), we found that *dcy* mRNA is strongly enriched in the midgut and heads of adult flies (Fig. 1*B*).

The *dcy* gene encodes one transcript for a 477-aa protein with a predicted molecular mass of 55.9 kDa. This protein was initially named Drosocrystallin because of its strong expression in the *Drosophila* compound eye, where it is thought to be a structural component of the corneal lens (18, 19). Dcy contains a signal peptide and a chitin-binding domain. Its strong expression in the midgut suggested that Dcy could be a component of the PM. To address this hypothesis, we examined the localization of Dcy in the midgut with an anti-Dcy serum. The immunostaining analysis showed that a signal is observed on the PM with the anti-Dcy serum in wild-type intestines, suggesting that Dcy protein is a component of the PM in adult *Drosophila* (Fig. 1*C*).

Loss of *dcy* Compromises PM Permeability. To gain insight into the physiological role of *dcy*, we used a *Drosophila* strain, $dcy^{MB08319}$ (referred to as dcy^1), with a *Minos* transposon inserted in the first intron of *dcy* (Fig. S1*A*). The amount of *dcy* mRNA in dcy^1 homozygous flies or in flies carrying the dcy^1 allele over a deficiency [*Df(2L)Exel6030*] was less than 10% of that of wild-type flies (Fig. S1*B*). Dcy^1 flies fail to induce any *dcy* expression after oral infection with *Ecc15* (Fig. 1*A*). In addition, immunostaining of midgut sections from dcy^1 adults with the anti-Dcy serum gave only a faint signal in the PM (Fig. 1*C*). These results indicate that dcy^1 is a strong loss-of-function mutation of *dcy*. We also generated a precise excision line of dcy^1 by remobilization of the *Minos* transposable element. This line, referred to as dcy^{Rev}, expressed a normal level of *dcy* and was used in addition to the Oregon R strain as a wild-type control strain in the following experiments.

Dcy^1 homozygous mutants are viable, fertile, and do not show any external morphological defect. dcy^1 mutants are apparently not blind because they can orient themselves according to the light (Fig. S1*C*). Nevertheless, the dcy^1 have a shorter lifespan, starting to die 3 to 4 wk after emergence (Fig. S1*D*). Interestingly, transmission electron microscopy of sections of anterior midguts revealed that the thickness of the PM in dcy^1 adults is only approximately half that of control flies (Fig. 2*A*). We next investigated the effect of the dcy^1 mutation on the PM permeability by feeding adults with FITC-labeled dextran molecules of different sizes as described in ref. 13. The occurrence of FITC signals in close contact with the intestinal epithelium was interpreted as the result of molecules crossing the PM into the ectoperitrophic space (between the PM and epithelium). Fig. 2*B* and Fig. S2 show that ingested 70- and 150-kDa FITC-labeled dextran molecules were observed in contact with the epithelial cells of wild-type flies, whereas 500-kDa and almost all 250-kDa dextran molecules remained in the lumen because of their incapacity to cross the PM. Interestingly, we observed that in dcy^1 mutants, 250-kDa and a large proportion of 500-kDa molecules were found in close vicinity to the gut epithelium (Fig. 2*B*, *Right*, and Fig. S2). These results indicate that the PM of dcy^1 mutants tends to be more permeable than that of wild-type flies.

***Dcy* Is Required for the Defense Against Oral Infection with Entomopathogenic Bacteria.** We next used dcy^1 flies to analyze the contribution of the PM to the protection against oral bacterial infection. *P. entomophila* is a natural bacterial pathogen of *Drosophila* (20). Oral infection with *P. entomophila* at a high dose induces a strong local and systemic immune response in *Drosophila* but is still highly pathogenic because it quickly induces a blockage of food uptake and irreversible gut damage (10). Fig. 3*A* shows that dcy^1 mutants exhibit a higher susceptibility than wild-type to oral infection with a lethal dose of *P. entomophila*. The *dcy* mutants even succumb to the infection with a fourfold-diluted *P. entomophila* solution, which is not lethal for wild-type flies (Fig. S3*A*). Several lines of evidence indicate that this increased susceptibility is indeed due to the *dcy*

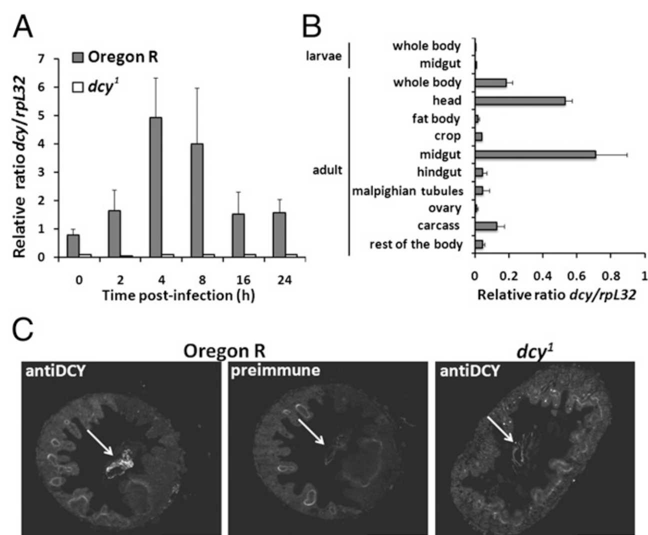

Fig. 1. *Dcy* expression is induced in the midgut upon oral bacterial infection. (*A*) *Dcy* expression upon *Ecc15* oral infection in wild-type and *dcy* mutant flies. *Dcy* mRNA was measured by real-time qPCR in whole flies at indicated time points, and results are shown as a relative *Dpt/rpL32* ratio. (*B*) Real-time qPCR analysis of *dcy* mRNA expression from the indicated tissues of wild-type *Drosophila*. Note that adult carcasses do not include gut. Data are representative of three (*A*) or two (*B*) independent experiments (shown are error bars). (*C*) Transversal sections of wild-type or *dcy* adult anterior midgut were analyzed by immunostaining with an anti-Dcy serum. Arrows indicate the PM (Scale bars, 50 μm.)

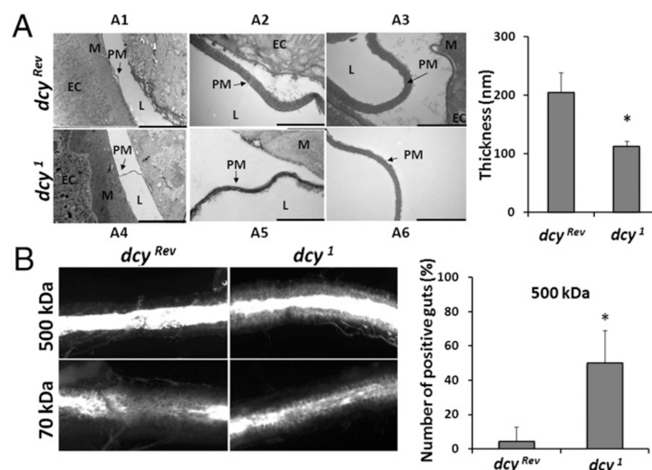

Fig. 2. The *dcy* mutation induces PM defects. (*A*) *Left*: Ultrathin sections of adult anterior midguts derived from wild-type or dcy^1 mutant flies were observed by transmission electron microscopy. *A2* and *A5* are magnified views of *A1* and *A4*. *A4* and *A6* show another section at high magnification. Arrows indicate the PM. M, mucus; EC, enterocytes; L, lumen with ingested food. (Scale bars, 10 μm in *A1* and *A4*, 1 μm in *A2*, *A3*, *A5*, and *A6*.) *Right*: Quantitative measurements of the thickness of the PM in wild-type or dcy^1 flies. Data show means and SEs from six and nine different midgut sections for the control and the mutant, respectively. (*B*) Dextran-feeding assay of wild-type or dcy^1 flies. Adult flies were fed with 70-kDa or 500-kDa FITC-labeled dextran beads. Guts were dissected and examined under a fluorescence microscope. The FITC signal is retained in the lumen if the dextran beads cannot pass through the PM. The FITC signal is observed in contact with epithelial cells (indicated as positive) if beads can cross the PM. Bar graph shows the number of "positive" guts for each genotype when 500-kDa molecules were fed. Means and SEs from four independent experiments are shown. *$P < 0.05$.

mutation and not to the genetic background. First, revertant dcy^{Rev} flies do not show the increased susceptibility, and *Df(dcy)*/dcy^{1} flies exhibit the same phenotype as the homozygous dcy^{1} mutants (Fig. 3*A*). Second, another genetic background, a *y,w, Diptericin-lacZ, Drosomycin-GFP;* dcy^{1} line, has the same susceptibility as dcy^{1} flies (Fig. S3*B*). Third, an *in vivo RNAi* silencing of *dcy* in the intestine using a midgut-specific GAL4 driver (genotype: *NP1-GAL4; UAS-dcy-IR*) also results in higher susceptibility to *P. entomophila* (Fig. S3*C*). Fourth, overexpression of *dcy* in the midgut using the *NP1-GAL4* driver (genotype: dcy^{1}/dcy^{1}; *NP1-GAL4/UAS-dcy*) rescued the dcy^{1} susceptibility phenotype (Fig. 3*B*). Fig. 3*C* shows that the dcy^{1} mutants also succumb more rapidly than wild-type when orally infected with *Serratia marcescens Db11*, another entomopathogenic bacterium (7). Finally, dcy^{1} mutant flies have a wild-type resistance to *Ecc15*, a nonlethal Gram-negative bacterium, upon septic injury (Fig. S3*D*), indicating that the survival phenotype of the dcy^{1} mutant upon oral bacterial infection is not caused by a general weakness of dcy^{1} mutant flies.

Both resistance and tolerance mechanisms contribute to maintain gut integrity upon bacterial infection. Resistance mechanisms involve the activation of various immune responses that directly target pathogens, whereas tolerance mechanisms improve the capacity to survive infection without acting on bacterial elimination. (21). We observed that the number of *P. entomophila* detected in the gut 18 h after infection (when flies start to die) was roughly similar in dcy^{1} and wild-type (Fig. 3*D*). This result indicates that the dcy^{1} mutation does not affect bacterial clearance but rather the tolerance of flies to *P. entomophila* infection. This is consistent with a role of the PM in protecting the gut epithelium from lethal infection. This also indicates that the dcy^{1} phenotype was not caused by a feeding defect because dcy^{1} and wild-type flies ingested the same quantity of *P. entomophila*, as determined by cfu assay at 30 min after ingestion (Fig. 3*D*). Moreover, the triglyceride and glycogen stores of the dcy^{1} unchallenged mutants were not significantly different from wild-type (Fig. S4), indicative of normal digestive capacity.

Together, our results show that expression of *dcy* in the midgut is required for defense against oral infection with entomopathogenic bacteria.

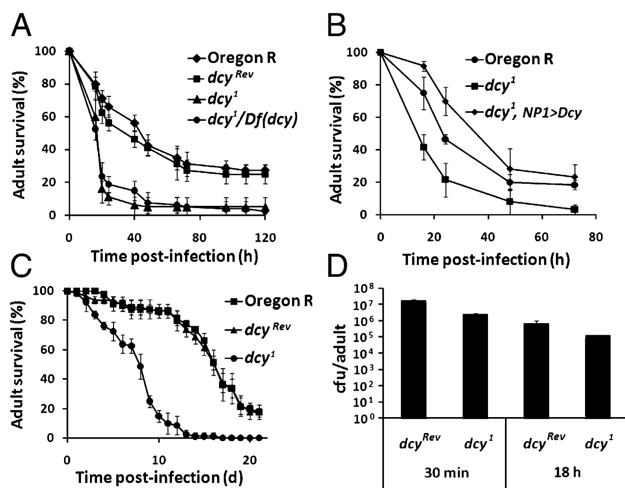

Fig. 3. Dcy is required for protection against oral infection with entomopathogenic bacteria. (*A*) Survival analysis of wild-type, dcy^{Rev}, homozygous dcy^{1}, and dcy^{1}/*Df(dcy)* hemizygous flies upon oral infection of *P. entomophila*. Means and SEs of four independent experiments are shown ($P <$ 0.0001, log–rank test). (*B*) Survival analysis of wild-type, homozygous dcy^{1}, and the dcy^{1} *mutants* expressing the *dcy* gene in the midgut upon oral infection of *P. entomophila*. Means and SEs of three independent experiments are shown ($P < 0.0002$, log–rank test). (*C*) Survival analysis of wild-type, dcy^{Rev}, and homozygous dcy^{1} flies upon oral infection with *S. marcescens Db11*. Graphs show the means of 60 flies, bars show the SE. This experiment was repeated three times and yielded similar results ($P < 0.0001$, log–rank test). (*D*) Bacterial persistence in wild-type and dcy^{1} flies. Bacterial persistence in dcy^{1} and dcy^{Rev} at 30 min and 18 h upon oral infection with *P. entomophila* (OD_{600} = 100), as the number of cfu per fly. No difference was observed between the two strains. Each histogram corresponds to the average of three independent experiments.

Enhanced Activation of the Imd Pathway in the *dcy* Mutant. The Imd pathway regulates antimicrobial peptide production in the gut and plays a role in the resistance against both *P. entomophila* and *S. marcescens* (6–8). This prompted us to analyze the effect of the dcy^{1} mutation on the Imd pathway. For this, we compared the expression of *Diptericin* (*Dpt*), an antibacterial peptide gene tightly controlled by the Imd pathway, in dcy^{1} and wild-type flies. Real-time qPCR revealed that dcy^{1} flies show a stronger induction of *Dpt* in the gut upon oral infection with *P. entomophila* (Fig. 4*A*), notably at early time points of infection. This higher induction was not specific to *P. entomophila* because similar results were obtained when flies were fed with the nonlethal strain *Ecc15* (Fig. S5). *P. entomophila* leads not only to a local but also to a strong systemic fat body immune response (20). Both real-time qPCR and X-gal staining with flies carrying a *Dpt-lacZ* reporter gene revealed higher levels of systemic *Dpt* expression in dcy^{1} mutant flies compared with wild-type upon *P. entomophila* infection (Fig. 4 *A* and *B*). The observation that the dcy^{1} mutation leads to a stronger overall activation of the Imd pathway in infected flies indicates that the PM influences bacterial sensing in the gut.

***Dcy* Mutation Affects Neither Resistance to ROS Nor Gut Repair Mechanisms.** Recent studies have demonstrated that the activation of epithelium renewal is required in the gut to compensate for damage caused by infectious agents. (9, 10, 22). Along this line, infection with high doses of *P. entomophila* leads to a loss of gut integrity, suggesting that virulence factors of this entomopathogen disrupt epithelium renewal through excessive damage to the gut. This raised the possibilities that the *dcy* mutant might be more sensitive to the oxidative burst associated with infection and/or has a compromised gut repair ability, either of which could explain the susceptibility of the *dcy* mutant to *P. entomophila*. To examine these possibilities, we orally administrated paraquat, a potent inducer of ROS, or bleomycin, a DNA-damaging agent that damages the gut, thus increasing stem cell proliferation (10, 23). Both compounds have molecular sizes (paraquat, 0.257 kDa; bleomycin, 1.4 kDa) small enough to easily cross the PM. We observed that dcy^{1} flies show survival rates similar to wild-type flies upon feeding with either paraquat or bleomycin (Fig. S6 *A* and *B*).

To determine whether the dcy^{1} mutation impacts gut repair, we quantified the level of epithelial renewal in flies upon infection with the strain *Ecc15*. We choose this strain because, unlike *P. entomophila*, oral infection with *Ecc15* triggers a high level of epithelial renewal without affecting flies' viability. We first examined the level of epithelium renewal itself upon *Ecc15* oral infection by counting the number of dividing cells along the midgut using an anti-phosphohistone H3 (anti-PH3) antibody as an indicator of mitotic activity (10). No difference was observed between wild-type and dcy^{1} flies (Fig. S7*A*). Signals from both JAK-STAT and EGFR pathways control stem cell proliferation and thereby gut repair and homeostasis after oral infection (16). We monitored the activation of these pathways by quantifying transcripts for *upd3*, *Socs36E* (JAK-STAT pathway), and *Keren* and *argos* (EGFR pathway) upon oral infection with *P. entomophila*. Fig. S7*B* shows that the *dcy* mutants express wild-type levels of these genes in response to *Ecc15* infection.

These results indicate that the dcy^{1} mutation does not induce a higher sensitivity to oxidative burst and does not affect the ability to repair the gut upon damage.

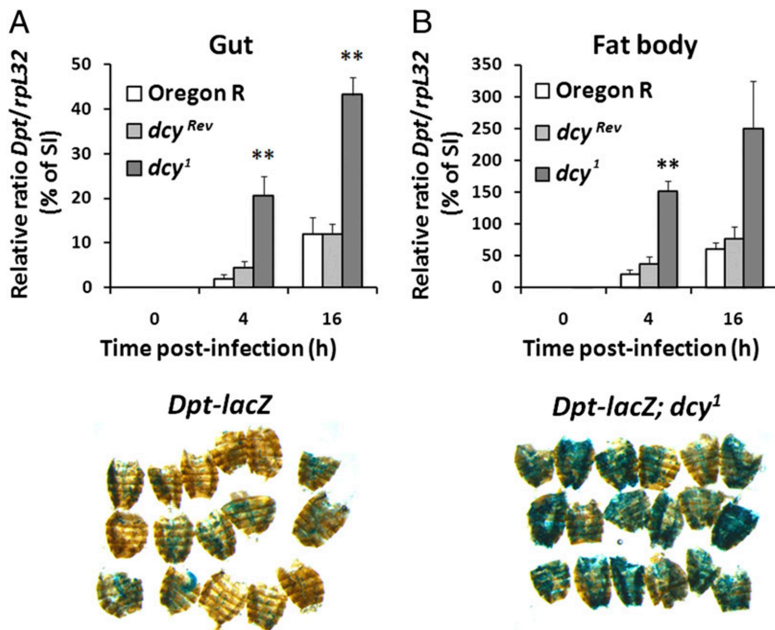

Fig. 4. *Dpt* expression upon oral bacterial infection is higher in dcy^1 flies compared with wild-type flies. (*A*) *Dpt* expression in the midgut (*Left*) or the fat body (*Right*) of wild-type and dcy^1 flies upon oral infection with *P. entomophila* was measured by real-time qPCR at the indicated time points. Data are the mean of four independent experiments, and error bars show the SE. $^{**}P < 0.01$ vs. dcy^{Rev}. Results are shown as a percentage of the *Dpt*/*rpL32* ratio normalized to the levels observed in wild-type flies collected 8 h after septic injury (SI) with *P. entomophila*. (*B*) β-Galactosidase staining reveals *lacZ* gene expression in the fat body of wild-type or dcy^1 flies carrying a *Dpt-lacZ* reporter. Flies were collected 4 h after oral infection with *P. entomophila*.

Increased Susceptibility of *dcy* Mutants to Ingested Bacterial Toxin. It has been proposed that the PM is a barrier protecting the midgut epithelium from secreted bacterial virulence factors such as toxins or proteases (12). We therefore speculated that toxic compounds secreted by *P. entomophila* could have a more damaging effect in the dcy^1 mutant owing to the higher permeability of the PM. Membrane-filtered extracts from sonicated *P. entomophila* cells were fed to wild-type and dcy^1 adults. We found that up to 80% of the dcy^1 mutants succumbed to this treatment, whereas no lethality was observed in wild-type flies fed with the same extract (Fig. 5*A*). Virulence factors of *P. entomophila* required for *Drosophila* infection include a secreted metalloprotease (AprA) that protects against antimicrobial peptides, and a pore-forming toxin named Monalysin that participates in the damage to intestinal cells (6, 24). Both AprA and Monalysin are regulated by the GacS-GacA two-component system that regulates the production of secreted proteins and metabolites (6). Fig. 5*B* shows that a *P. entomophila* extract from a *gacA* mutant no longer kills the dcy^1 mutant flies, whereas extracts from an *aprA* mutant retain their wild-type pathogenicity on dcy^1 flies (Fig. 5*C*). Interestingly, extracts from the *monalysin (mnl)* mutant *P. entomophila* less efficiently kill dcy^1 mutant flies (Fig. 5*D*), implying that this pore-forming toxin is partially responsible for the killing activity of the extract. The observations that (*i*) Monalysin contributes to the killing activities of the extract and (*ii*) the dcy^1 mutation increases the susceptibility to the *P. entomophila* extract indicate that the PM provides protection against ingested toxins, especially pore-forming toxins.

Discussion

Proteomic analyses have revealed the complexity of the insect PM that is composed of chitin microfibrils embedded in a matrix of proteins and glycoproteins (12, 25, 26). PM-associated proteins include mucins and peritrophin proteins. The *Drosophila*

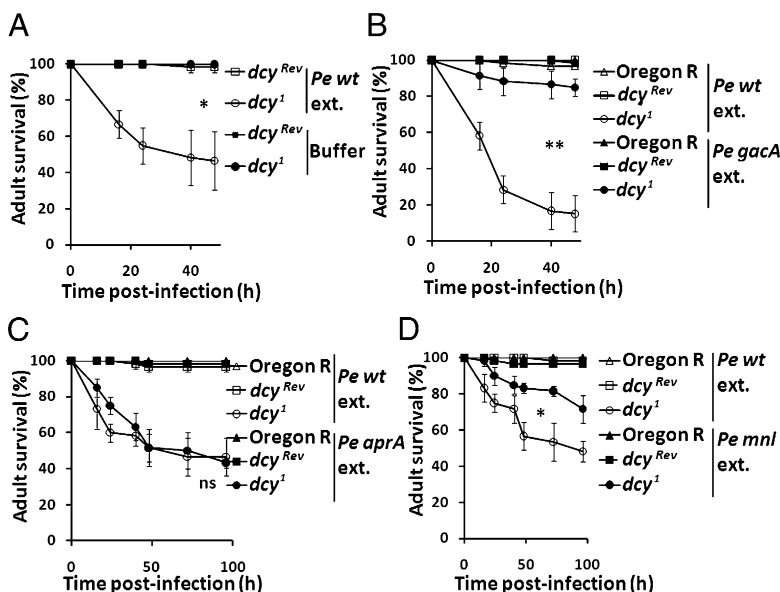

Fig. 5. Dcy^1 flies succumbed to ingestion of a *P. entomophila* extract. (*A*) Survival analysis of dcy^{Rev} and dcy^1 flies upon feeding of the extract from *P. entomophila* (*Pe* extract) or buffer (PBS 1% Triton X-100). (*B*) Survival analysis of wild-type, dcy^{Rev}, and dcy^1 flies upon feeding with extracts derived from wild-type and *gacA P. entomophila* derivatives. (*C*) Survival analysis of wild-type, dcy^{Rev}, and dcy^1 flies upon feeding with extracts derived from wild-type and *AprA P. entomophila* derivatives. (*D*) Survival analysis of wild-type, dcy^{Rev}, and dcy^1 flies upon feeding with extracts derived from wild-type and *monalysin* (*mnl*) *P. entomophila* derivatives. In *A*–*D*, graphs show the means of 60 flies, and bars show the SE. These experiments were repeated three times and yielded similar results ($^{*}P < 0.0001$, $^{**}P < 0.005$, log–rank test. ns, not significant).

genome contains approximately 25 genes encoding chitin-binding domain proteins and 36 genes encoding mucin-like proteins (27). To date, none of these genes have been studied in the context of the PM, and little is known about the role of mucus and the PM in *Drosophila* gut homeostasis and immunity. Here we show that the Dcy protein, a putative component of the eye lens of *Drosophila*, contributes to adult PM formation. Although, we cannot exclude that Dcy also exist as free molecules in the gut, four lines of evidences support that Dcy is an integral component of the PM: (*i*) the presence of a chitin-binding domain, suggesting that Dcy associates with chitin fibrils of the PM; (*ii*) the staining of the PM using an anti-Dcy antibody; (*iii*) the thinner PM observed in dcy^1 mutants; and (*iv*) the higher permeability of the PM in dcy^1 mutants. Because *dcy* is expressed in the midgut and not in the cardia, it is likely that this protein is directly incorporated to the PM after its synthesis. The observation that a strong loss-of-function mutation in *dcy* reduces the PM width by half and increases its permeability to larger molecules indicates that despite the high number of structural proteins associated with the PM, Dcy is an essential component of the PM. Dcy cannot be considered *sensu stricto* as a peritrophin, owing to the absence of characteristic cystein arrangement in its chitin-binding domain. The dual function of this protein in both eye and gut is intriguing. Of note, no clear Dcy paralog is encoded by the *Drosophila* genome. In addition, no homolog can be found outside the Drosophilidae family, suggesting that Dcy has evolved to fulfill a function specific to this clade.

Using the dcy^1 mutation, we were able to indirectly assess a role for the PM in *Drosophila* host defense. Our observation that *dcy* mutants are highly susceptible to infection with *P. entomophila* and *S. marcescens* points to a protective role of the PM in host defense against entomopathogenic bacteria. We observed that ingestion of a *P. entomophila* extract is sufficient to induce lethality in dcy^1 mutant flies but not in wild-type flies. This supports a role of the PM in limiting the diffusion of a bacterial toxin. Interestingly, a *P. entomophila* extract from the pore-forming toxin *monalysin*-deficient strain seems less toxic to *dcy* flies. This indicates that the PM provides an effective protection against the action of this pore-forming toxin. β-Pore-forming toxins such as Monalysin have the ability to multimerize into circular polymers, a step required for pore formation in targeted cells (28). This suggests that the PM could function as a sieve blocking the action of this class of toxins. A role of the PM in the protection against pore-forming toxins is also supported by studies in other insects. Hayakawa et al. (29) showed that *Bombyx mori* is sensitive to the Cry1Aa toxin and resistant to Cry1Ac, both insecticidal toxins of *Bacillus thuringiensis*. This difference correlates with the capacity of Cry1Aa to pass through the PM faster than Cry1Ac in an in vitro assay. It was also reported that the activity of Cry1Ac toxin on *Helicoverpa armigera* larvae is enhanced by *B. thuringiensis* Enhancin, a metalloprotease that degrades PM-associated mucins (30). The involvement of a pore-forming toxin in *P. entomophila* virulence, together with the well-characterized action of *B. thuringiensis* cytotoxin Cry, have recently led to the notion that pore-forming toxins constitute an efficient arm to promote bacterial colonization of the insect gut (24, 31). Our present studies suggest that the PM provides an important barrier to counteract the action of this class of toxins.

The observation that *dcy* as well as several peritrophin genes are induced upon ingestion of bacteria (16) also points to the existence of active mechanisms that reinforce the role of the PM barrier during infection. This indicates that the PM is not just a passive physical barrier but can be remodeled during gut infection. However, our study does not address whether PM protection against *P. entomophila* pore-forming toxin is mediated by its impermeability to Monalysin or by its capacity to bind and sequester this toxin. In support of the second hypothesis, Abedi and Brown (32) discovered that the PM excreted by *Aedes aegypti* larvae that are resistant to dichlorodiphenyltrichloroethane was laden with the insecticide. This finding led to the notion that this matrix may play a role in sequestering and possibly detoxifying ingested xenobiotics.

Production of ROS in the gut by Duox in response to bacteria inflicts damage to the intestinal epithelium that is repaired through stem cell proliferation (10). Although some of these ROS compounds could be inactivated by antioxidant enzymes (33), it has been proposed that the PM could serve as a "sacrificial antioxidant" through the scavenging of ROS (34, 35). In opposition to this idea, we did not observe a higher susceptibility of *dcy* mutants to ROS produced by paraquat. This does not rule out a role of the PM as antioxidant but suggests that in our infection model the main defensive role of the PM is to limit the action of bacterial toxins. Moreover, the gut repair capacity through epithelium renewal is not affected by the *dcy* mutation in flies orally infected with *Ecc15* or flies that ingested Bleomycin, thus reinforcing the idea that the PM protection is specific for a certain type of threat, such as that linked to pore-forming toxins.

Recent studies in *Drosophila* have revealed that multiple regulatory mechanisms are required to precisely control the level of Imd pathway activity in the gut. These include Pirk, a protein interacting with PGRP-LC and with amidase PGRPs, which both restrict the activation of the immune pathway by indigenous flora (5, 36). Here, we observed that disruption of the PM affects the level of Imd pathway activity in response to infection. Dcy^1 mutants exhibit enhanced gut and systemic immune responses to Gram-negative bacteria. This indicates a role of the PM in the fine-tuning of Imd pathway activity during bacterial infection. An attractive hypothesis is that the PM could limit the diffusion from the gut lumen to epithelial cells of peptidoglycan, the bacterial elicitor recognized by the Imd pathway.

In conclusion, our studies ascribe important functions to the PM in *Drosophila* host defense against bacteria by limiting the effect of bacterial toxins and reducing Imd pathway activity. The importance of the PM function is even underestimated in our study because the dcy^1 mutation reduces but does not eliminate the PM. Our study is also in line with those in vertebrates that revealed the key role of mucus in gut homeostasis and mucosal immunity (37). Indeed, deletion of the large gel-forming mucin Muc2 in mice allows the direct contact of bacteria with the epithelia cells, thus provoking colon inflammation. It now seems that both mucus in mammals and PM in insects provide an important protection against the action of pathogens and influence the immune reactivity of the digestive tract. A better comprehension of the physiological role of the PM is essential to understand insect gut homeostasis. Moreover, we must keep in mind that the PM is an attractive target for insect pest management strategies (11, 12). We expect that this study will open the route for a genetic dissection of PM function in *Drosophila* that could be useful in other insects of economic or global health importance.

Materials and Methods

Fly Stocks. Oregon R flies were used as wild-type flies. Dcy^1 (MB08319, Fig. S1) and *Df(2L)Exel6030* were obtained from the Bloomington *Drosophila* Stock Center. Canton S, w^{1118}, $Relish^{E20}$ (Rel^{E20}), *NP1-Gal4*, and *Diptericin-LacZ* fly lines are described in ref. 16. The *UAS-CG16963-IR* RNAi line from the Vienna *Drosophila* RNAi Stock Center and *UAS-yellow* (Bloomington Center) or w^{1118} were used as control. A full-length cDNA of *dcy* (CG16963_cDNA gold RH66281 from the *Drosophila* Genomics Resource Center) was inserted in the pENTR Gateway entry clone (Invitrogen) and then subcloned in the pTW transgenesis vector used for generating transgenic flies according to standard procedures. Fly line carrying the transgene on the third chromosome was established and used as *UAS-dcy*. F1 progeny carrying both the *UAS* construct and the *Gal4* driver were transferred to 29 °C at late pupal stage for optimal efficiency of the *UAS/Gal4* system. To obtain a revertant of dcy^1, the *Minos*-element *MB08319* was mobilized by a *Minos* transposase source (38), and precise excision line (referred to as dcy^{rev}) was isolated. *Drosophila* stocks and

crosses were maintained at 25 °C in tubes containing standard fly medium (maize flour, dead yeast, agar, and fruit juice) devoid of living yeast.

Bacterial Stocks and *P. entomophila* Protein Extracts. *P. entomophila* L48 (20) was grown in LB for all experiments. *P. entomophila* mutated for the *gacA*, *aprA*, and *mnl* are described elsewhere (6, 24). The *Ecc15* strain was described previously (20). They were grown at 29 °C and allowed to reach the stationary phase. Cells were then concentrated at OD_{600} = 200 except when indicated. The solution was added. *S. marcescens* strain *Db11* (7) was grown at 37 °C and used as pellets of OD_{600} = 200. Pellets were not washed before use. For *P. entomophila* extracts, stationary phase cultures of wild-type and mutant *P. entomophila* were concentrated by centrifugation. The cell pellet was washed with PBS and adjusted to OD_{600} = 200 in PBS with 1% Triton X-100. The pellets were sonicated, recentrifuged, and filtered with a 0.22-μm membrane (Millipore).

Infection and Survival Assays. Septic injuries were performed by pricking adults in the thorax with a thin needle dipped into a concentrated bacterial pellet. For oral infection, female flies were starved for 2 h at 29 °C and then placed in a fly vial with food solution. The food solution was obtained by mixing a pellet of bacteria (OD_{600} = 200, corresponding to 1.3×10^{11} bacteria/mL), solution of paraquat (10–50 mM, Sigma), or 500 μg/mL bleomycin (Sigma) with a solution of 5% sucrose (1:1), added to a filter disk that completely covered the surface of standard fly medium. Flies were maintained at 29 °C, and mortality was monitored as at different time points. Survival assays were performed with 60–80 flies for each genotype.

Antibodies, Immunohistochemistry, and X-Gal Staining. Anti-PH3 staining was performed as previously described (16). An Anti-Dcy antibody was raised by immunizing rats with the carboxyl end of Dcy protein (amino acid positions 402–477), which was expressed in *Escherichia coli* as a fusion protein with GST and affinity-purified. Immunohistochemistry, electron microscopy, and X-gal staining are described in *SI Materials and Methods*.

Dextran Feeding Assay. FITC-dextran beads (Sigma) were dissolved in 2.5% sucrose and filtrated with Sephadex G-10 (GE Healthcare) and used for feeding experiments. Female flies were starved for 2 h at 29 °C in an empty vial and then placed in a normal fly vial covered with a filter paper soaked with the dextran solution. After 15 min at 29 °C, guts were dissected in PBS, and FITC signal was observed under a fluorescent microscope (Zeiss). Images were captured with a Leica DFC300FX camera and the Leica Application Suite.

Real-Time qPCR. Total RNA was extracted from whole flies, cuticles of flies as fat bodies, dissected midguts, or other tissues by using TRIzol (Invitrogen). Real-time qPCR was performed using SYBR Green I (Roche) on a LightCycler 2.0 as described previously (16). The amount of mRNA detected was normalized to *rpL32* control values. Primers used to monitor mRNA quantification can be obtained on request.

Statistics. Statistical analyses were done by Student *t* test or log–rank test, and *P* values of <0.05 were considered significant.

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Supporting Information

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SI Materials and Methods

Antibodies, Immunohistochemistry, and X-Gal Staining. For immunohistochemistry, guts were dissected in PBS, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS, and washed in PBS. The guts were treated in an ascending series of ethanol concentrations for dehydration and embedded in LRWhite (London Resin). The guts were cut at 500 nm with an ultramicrotome (Leica) and attached to slide glasses. The samples were then stained with anti-Dcy serum as described by Micheva et al. (1). A secondary staining was performed with Alexa 594 coupled with an anti-rat antibody (Invitrogen) and mounted with DABCO (Invitrogen). Samples were scanned with an LSM 700 confocal laser microscope (Zeiss). β-Galactosidase was visualized by X-gal staining, as previously described (2), then mounted in a 50:50 mix of ethanol and glycerol. Images were captured with a Leica DFC300FX camera and the Leica Application Suite.

Electron Microscopy. *Drosophila* adults were dissected into PBS, and the guts were immediately fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in PBS for 4 h at 4 °C. The samples were rinsed three times in 0.1 M cacodylate buffer, then postfixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide solution in 0.1 M cacodylate buffer for 40 min at room temperature, followed by 1% osmium tetroxide solution in 0.1 M cacodylate buffer for 40 min at room temperature. The samples were then treated with 1% uranyl acetate in water for 40 min at room temperature. Dehydration of the guts was performed in an ascending series of ethanol concentrations, and then the samples were embedded in Durcupan (Fluka). Guts were cut at 50 nm for transmission electron microscopy with a Leica ultramicrotome. Ultrathin sections were contrasted with lead citrate and observed with a transmission electron microscope.

Bacterial Persistence. Bacterial persistence was measured in wild-type and *dcy*[1] mutant flies by plating appropriate dilutions of homogenates of five surface-sterilized adults on LB-agar plates.

Fat and Glycogen Assays. For fat assay, 10 males were homogenized in 0.2 mL PBS 0.1% Tween-20. After heat-inactivation for 10 min at 70 °C, the lysate was centrifuged for 5 min at 15,700 × *g*. The supernatant (0.02 mL) was subjected to fat quantification using a Free Glycerol Determination Kit (Sigma). Fat amount was determined by adding the quantity of glycerol to the amount of triglycerides. For glycogen assay, 10 male flies were homogenized in 1.0 mL of cold lysis buffer [0.01 M KH_2PO_4 and 1 mM EDTA (pH 7.4)], and the lysate was centrifuged for 2 min at 400 × *g*. The supernatant (0.025 mL) was used to measure glycogen using a Glucose (HK) Assay Kit (Sigma), in which 1 U/mL of amyloglucosidase was supplemented. This reaction was blanked against a reaction in which amyloglucosidase had not been added. All reactions were carried out in 96-well plates with 0.2 mL reagent per well.

Phototaxis. A tube apparatus was used for testing flies' vision. A fly tube coated with black tape was joined to an empty fly tube. Forty female flies were placed in the dark tube, and a source light was applied at the distal end of the empty tube for 5 min. At the end of the test, the distribution of flies in each tube was quantified.

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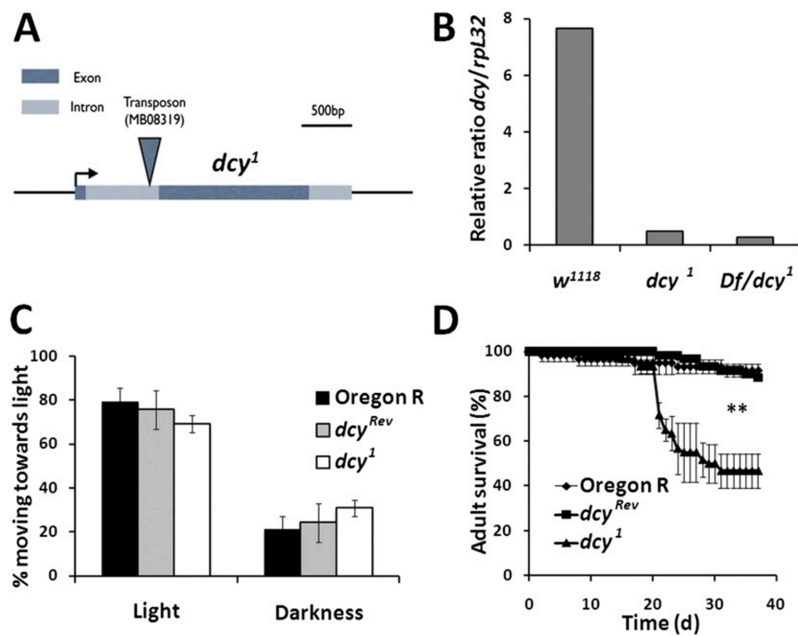

Fig. S1. *Dcy*^{1} is a strong loss-of-function mutant affecting the *drosocrystallin* gene. (*A*) Schematic representation of the *dcy* locus with the position of the *MB08319 Minos* transposon. (*B*) Real-time quantitative PCR analysis of *dcy* mRNA in *white*^{1118}, *dcy*^{1} homozygous, and *Df(dcy)/dcy*^{1}hemizygous flies. (*C*) Phototaxis of the *dcy*^{1} flies. (*D*) Lifespan analysis of unchallenged flies reveals an increase in mortality rate of *dcy*^{1} flies ($P < 0.0001$). Each survival curve corresponds to at least three independent experiments of three tubes of 20 flies each. *P* values were calculated using a log–rank test.

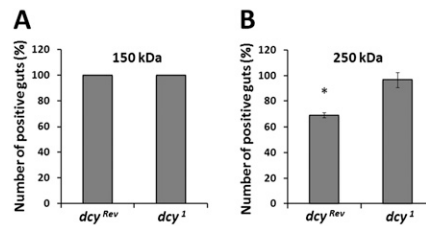

Fig. S2. Dextran-feeding assay of wild-type or *dcy*^{1} flies. Adult flies were fed with (*A*) 150-kDa or (*B*) 250-kDa FITC-labeled dextran beads. Guts were dissected and examined under a fluorescence microscope. The FITC signal is retained in the lumen if the dextran beads cannot pass through PM. The FITC signal is observed in contact with epithelial cells (indicated as positive) if beads can cross the PM. Bar graph shows the number of "positive" guts for each genotype when dextran molecules were fed. Means and SEs from three independent experiments are shown. $^*P < 0.05$.

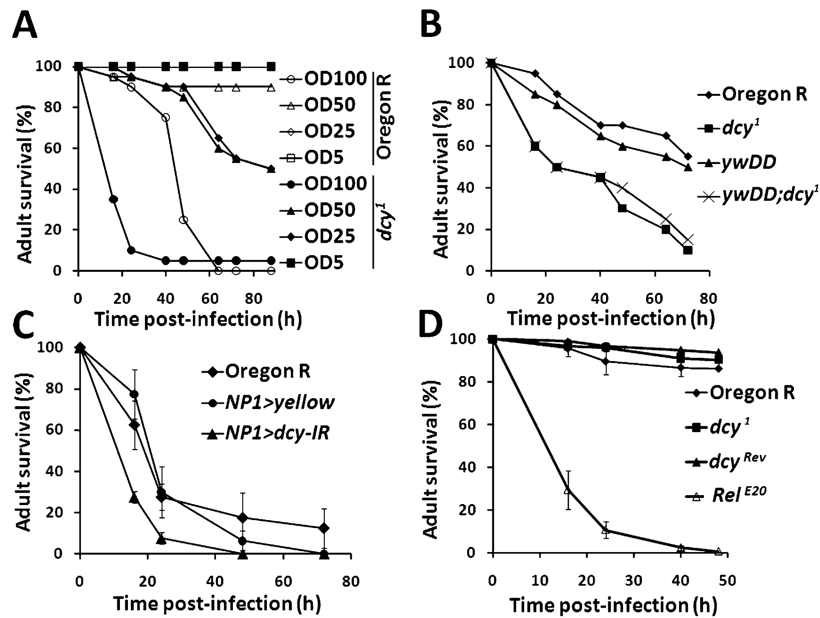

Fig. S3. Survival assays of the *dcy¹* mutant upon various challenges. (*A*) Survival analysis of wild-type and homozygous *dcy¹* flies upon oral infection with *P. entomophila* at different concentrations: OD of the *P. entomophila* feeding solution was 100, 50, 25, or 5. This experiment was repeated three times and yielded similar results ($P < 0.0003$, log–rank test). (*B*) Survival analysis of wild-type, *dcy¹*, *y,w, Dpt-LacZ, Drs-GFP* (referred to as *ywDD*), and *ywDD;dcy¹* flies upon oral infection with *P. entomophila* (OD_{600} = 200). This experiment was repeated three times and yielded similar results ($P = 0.0207$, log–rank test). (*C*) Survival analysis of wild-type, *NP1-Gal4 UAS-yellow* (*NP1>yellow*), and *NP1-Gal4 UAS-dcy-IR* (*NP1>dcy-IR*) flies upon oral infection with *P. entomophila* (OD_{600} = 200). Means and SEs of four independent experiments are shown ($P < 0.0003$, log–rank test). (*D*) Survival analysis of Oregon R, Canton S, *w¹¹¹⁸*, *Rel^E20^*,*dcy¹*, and *dcy^Rev^* flies upon septic injury with *Ecc15*. This experiment was repeated three times and yielded similar results (not significant).

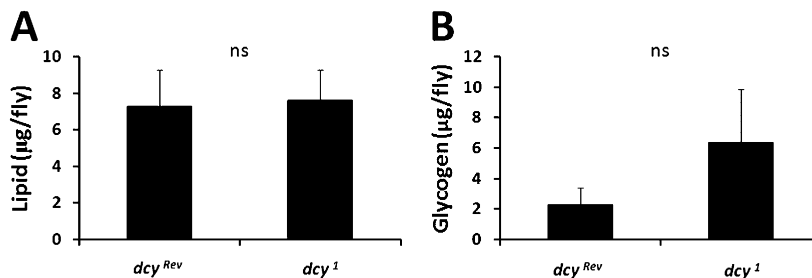

Fig. S4. *Dcy¹* mutation did not affect the triglyceride and glycogen stores. Quantification of fat (*A*) and glycogen (*B*) amount in *dcy¹* and *dcy^Rev^* flies. Amount of fat and glycogen are in micrograms per fly. This experiment was repeated three times and yielded similar results.

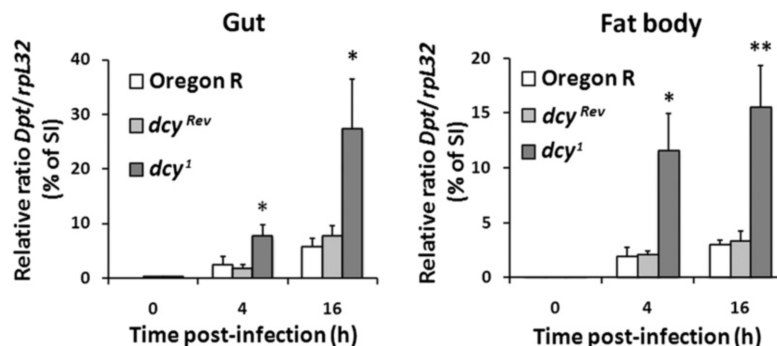

Fig. S5. *Dpt* expression upon oral *Ecc15* infection is higher in *dcy¹* flies compared with wild-type flies. *Dpt* expression in the midgut (*Left*) or the fat body (*Right*) of wild-type and *dcy¹* flies upon oral infection with *Ecc15* was measured by real-time quantitative PCR at the indicated time points. Data are the mean of four to seven independent experiments, and error bars show the SE. $*P < 0.05$, $**P < 0.01$ vs. Oregon R.

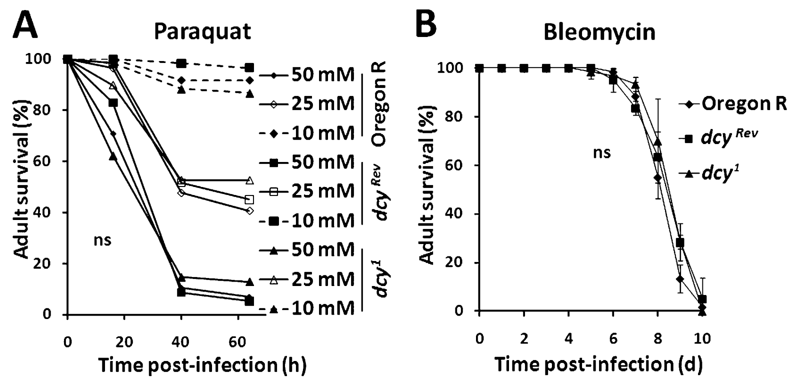

Fig. S6. *Dcy*[1] mutation does not affect resistance to damaging agents. (*A*) Survival analysis of wild-type, *dcy*[Rev], and *dcy*[1] flies upon feeding of paraquat as the indicated concentration. Data show means and SEs from three cohorts and are representative of two independent experiments. (*B*) Survival analysis of wild-type, *dcy*[Rev], and *dcy*[1] flies upon feeding of 250 μg/mL of bleomycin. Data are representative of two independent experiments. ns, not significant.

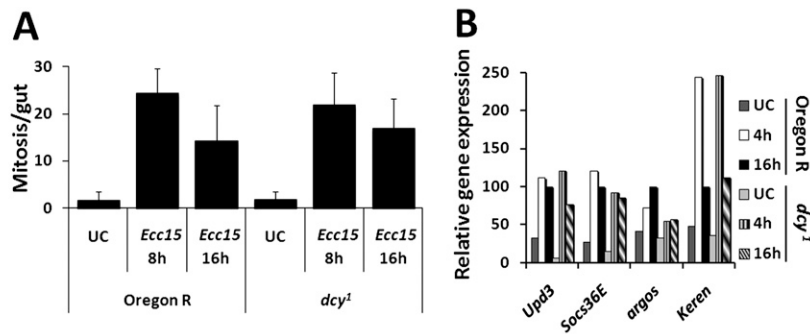

Fig. S7. *Dcy*[1] mutation does not affect gut repair in response to *Ecc15*. (*A*) Quantification of PH3-positive cells per midgut was monitored in wild-type and *dcy* mutant flies upon *Ecc15* oral infection. Mean values from three experiments (n = 15–20 guts each) ± SE are shown. (*B*) Real-time quantitative PCR analysis of gut extracts shows that genes regulated by the JAK/STAT (*upd3*, *Socs36E*) and EGFR (*argos*, *Keren*) pathways are induced at the same levels in wild-type and *dcy*[1] flies upon oral ingestion with *Ecc15*. Values are ratio over *RpL32* that were normalized to their maximum expression levels.